Kinetic aspects of the interaction of blood clotting enzymes

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THROMBOSIS ET DIATHESIS HAEMORRHAGICA

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Kinetic Aspects of the interaction of Blood Clotting Enzymes

III. Demonstration of an Inhibitor of Prothrombin Conversion in Vitamin K Deficiency

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Introduction

In the routine checking of patients under anticoagulant treatment a discrepancy has frequently been observed between the results of various clotting assays. Study of the published data on the clotting factor levels in long-term anticoagulant treatment shows that according to some authors the four vitamin K-dependent clotting factors (i. e. factors II, VII, IX, and X) drop to the same level, but that the level indicated by an over-all assay such as the thrombotest procedure (23) is lower than the levels found for the individual factors (13, 18).

Recently, we estimated that in steady state anticoagulation the level of any of the individual factors can be calculated as 2.5 times the percentage found from the thrombotest assay (15). Other authors, however, have reported a specific lowering of one factor relative to the others in long-term treatment with vitamin K antagonists (28) and it has been postulated that the percentage found from a thrombotest assay indicates this lowest level (24). Since we were unable to confirm this finding (12, 14), it appeared that different methods of estimation could lead to essentially different results, even for the level of only one of the clotting factors.

The results of kinetic studies on the clotting reaction occurring with use of the thrombotest reagent led us to assume that in vitamin K deficiency an inhibitor of this reaction must be present (5, 6). It could be shown that the prolongation of clotting time caused by the presence of this inhibitor results in an underestimation of the level of the clotting factors in the sample, especially when this sample is present in relatively undiluted form in the final reaction mixture. Therefore, this inhibitor had a marked influence in the thrombotest reaction, in which the plasma to be tested constitutes 10–15% of the final reaction mixture, but was not easily recognized in our assays of the individual clotting factors, where the plasma to be tested amounts to only 2.5% of the final reaction volume (see under Methods). Consequently, the latter tests must be fairly insensitive to this inhibitor.

It seems likely that the discrepancies in the published results were caused by variations in the influence of the inhibitor depending on dilution as well as by possible variations in sensitivity to this inhibitor inherent to the various specific assays used by the different authors.

Although we have not yet been able to obtain this inhibitor in a pure state, we think that at present the kinetic experiments have supplied sufficient evidence to permit recognition of the postulated inhibitor as a definite entity.

The use of the estimation of the inhibitor as a means of differentiating between vitamin K deficiency and liver damage in clinical laboratory practice has already been described (8).

On the basis of a hypothesis concerning the synthesis of vitamin K-dependent clotting factors, we gave the name *preprothrombin* to this inhibitor in our preliminary reports. Since there are indications that the inhibitor is related to other vitamin K-dependent clotting factors and not to factor II, and because other workers have meanwhile introduced a *prethrombin* (17), the term preprothrombin is apt to be very confusing. For this reason, this term will be replaced here by *P*rotein *I*nduced by *V*itamin *K A*bsence or *A*ntagonists (PIVKA).

Materials and Methods

Unless otherwise stated, *Dicumarol plasma* indicates a freshly pooled plasma from at least 10 patients under long-term anticoagulant treatment and under routine supervision by our thrombosis service[1]). This plasma was freed of platelets by centrifugation for 20 min at 20,000 g in a cooled super-speed centrifuge (Lourdes betafuge or Servall RC-2b). *Normal plasma* always indicates a pooled platelet-free plasma from at least 30 healthy normals (equal number of males and females, mean age around 30 years) spun platelet-free (20 min; 20,000 g; 4° C) and stored at −20° C in 1 ml portions. *Thromboplastin* indicates human brain thromboplastin prepared according to Owren and Aas (22).

Specific reagents used:

Factor II reagent:

a) Plasma from a case of severe congenital factor II deficiency, centrifuged and stored as the normal plasma pool; this plasma was kindly supplied by Dr. M. C. Verloop, University Hospital, Utrecht.

b) A mixture of equal parts of oxalated human serum and Barium Sulphate adsorbed bovine oxalated plasma, prepared according to Loeliger (11).

Factor V reagent was prepared according to Borchgrevink (3).

Factor VII reagent was plasma from a case of congenital factor VII deficiency, centrifuged and stored as the normal plasma pool.

Factor X reagent was plasma from (a) a congenitally factor X-deficient plasma or (b) plasma from a patient with an acquired factor X deficiency due to amyloidosis. These plasmas were kindly supplied by Dr. M. C. Verloop (University Hospital, Utrecht) and Dr. G. den Ottolander (Dijkzigt Hospital, Rotterdam), respectively.

Factor VII–X reagent was Seitz-filtered oxalated bovine plasma, prepared according to Bachmann (1).

All specific clotting tests were carried out in the same way: 0.1 ml reagent, 0.1 ml thromboplastin and 0.1 ml sample diluted $^1/_{10}$ with Michaelis buffer, were incubated for 30 sec, after which the reaction was started with 0.1 ml of $CaCl_2$ 25 mM.

The moment of clotting was determined with a Kolle hook, and the time between the commencement of the reaction and the moment of clotting was measured with a chronometer. All reactions were carried out at 37° C ± 0.05° C.

The thrombotest reaction was carried out with 0.25 ml Thrombotest reagent (Nyegaard, Oslo) to which 0.05 ml of plasma or a plasma dilution was added. The clotting time was again measured with a Kolle hook or in the coagulometer (29).

The buffer used for dilution was Michaelis buffer pH 7.4.

Dilution (D) will be defined as the ratio of final volume of a plasma sample over its original volume; therefore, undiluted plasma had a dilution factor of unity (D = 1). D = x means that x-1 volumes of diluent were added to one volume of plasma.

A t–D plot is a plot in which the clotting time (t_c) obtained with plasma at a known dilution (D) is plotted along the Y-axis and the dilution along the X-axis. When the term "clotting factor

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1) The large majority of these patients was treated either with phenprocoumon or with acenocoumarin.

concentration" is used in this context, we mean the concentration of the clotting factor that is rate-limiting in these clotting time estimations. Usually, this will be factor X (9). The concentration is expressed, as usual, as a percentage of the concentration in the normal plasma. In the t–D plots shown here, each point represents the mean of at least 16 determinations.

Theoretical Considerations

In a previous article (9) we have shown that the t–D plot obtained with a dilution of normal plasma in the thrombotest reagent is a straight line.

Furthermore, it was demonstrated in that article that unless the level of factor II or factor VII is significantly lower than the level of factor X, the clotting time obtained with the thrombotest reagent is determined by the level of factor X. This held regardless of whether the sample was diluted or undiluted.

In both *chronic* liver disease and *prolonged* vitamin K deficiency, factors VII and II are known not to show any tendency to drop below the level of factor X (12, 14). Therefore, the graph obtained in a t–D plot estimation with thrombotest may for all practical purposes be regarded as an experiment in which the dilution of *factor X* is plotted against the clotting time.

A t–D line shows two features of special importance: the slope of the line (α; expressed as the cotangent of the angle between this line and the abcissa) and the intercept of the line with the ordinate. This intercept indicates a point on the clotting time axis and therefore a clotting time, called *minimal clotting time* (t_{min}).

We have already shown (7) that the ratio of the slope (α_x) obtained with any given plasma to the slope (α_n) obtained with normal plasma indicates the amount of rate-limiting clotting factor (i. e. factor X) in the unknown plasma. In the following pages, α_n will be arbitrarily considered to equal 100; α_x then directly indicates the level of factor X in %.

The physical meaning of t_{min} is that this is the shortest clotting time obtainable by increasing the concentration of factor X. By extrapolation, we get t_{min} as the clotting time at *infinite factor X* concentration and at a level of factors II and VII that represents an excess of these factors. This means that all plasmas differing only in their factor X content should have the same t_{min}, because the factor X level is of no importance once the extrapolation to infinite factor X concentration has been carried out.

Since it has been shown that levels of factors II and VII equal to or higher than the level of factor X represent an excess of reactants, this statement can be extended to:

All plasmas with different factor X contents and with factor II and factor VII contents not significantly lower than their factor X level, have a t_{min} that is equal to the t_{min} of normal plasma, provided no modifiers of the reaction are present.

Under modifiers we understand here substances that have a specific influence on the reaction but that are not taken account in the above reasoning. Both accelerators and inhibitors fall into this category.

Ionic strength, pH, temperature, Ca^{++} ion concentration, etc. may also be regarded as modifiers of the reaction rate. Their influence is negated when estimations are performed under controlled conditions.

Experimental Results

Fig. 1 shows a t–D plot of a pooled plasma of 12 patients with chronic liver disease. Fig. 2 shows a t–D plot of a pooled plasma of 12 patients under prolonged anticoagulant treatment.

It is obvious that the minimal clotting time obtained with the Dicumarol plasma is markedly higher than that of normal plasma, whereas the minimal time of cirrhosis

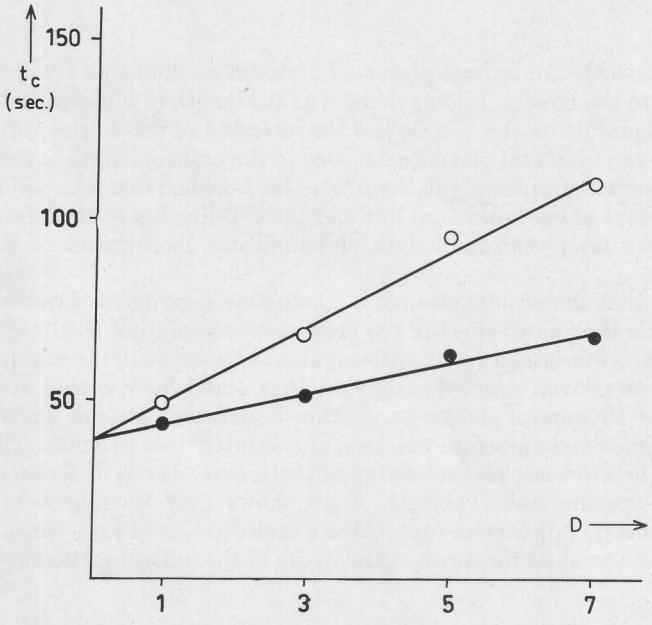

Fig. 1. *Thrombotest time-dilution plot of cirrhosis plasma.* ●———● –t Dplot of normal plasma; ○———○ t–D plot of a pooled plasma of 12 patients with chronic hepatic cirrhosis. t_{min} of both plasmas in 39 sec. The relation of the slopes of the two lines (α) indicates a factor X content of 40% for the cirrhosis plasma. For the cirrhosis plasma, I = 0.

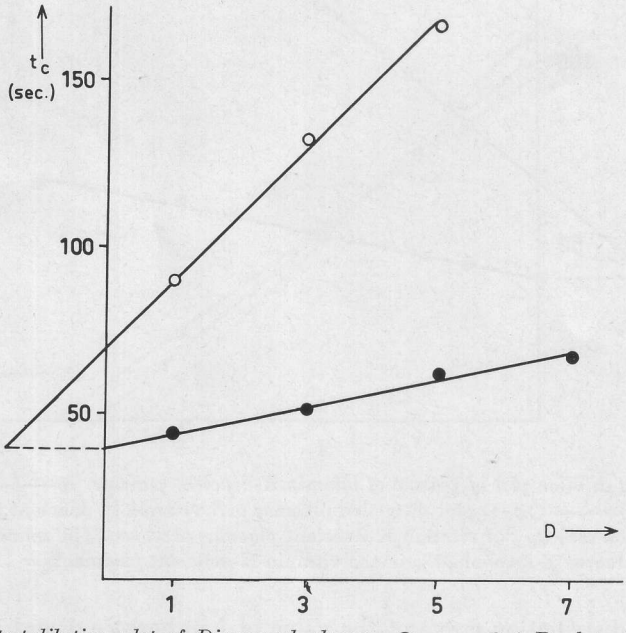

Fig. 2. *Thrombotest-dilution plot of Dicumarol plasma.* ●———● t–D plot of normal plasma. ○———○ t–D plot of Dicumarol plasma. t_{min} of normal plasma is 39 sec, t_{min} of Dicumarol plasma is 70 sec. The relation of the slopes (α) indicates a factor X level of 22% in the Dicumarol plasma. I = 1.6.

plasma is equal to that of normal plasma. Furthermore the value I will be mentioned in the legends to the figures, I being defined as the length of a horizontal line between t_{min} of normal plasma on the Y-axis and the intercept of this horizontal line with the t–D line of the experimental plasma expressed in the units of length of the X (-dilution) axis. I is represented graphically in the figures by a dashed line. I is said to be positive when the intercept of the horizontal line and the t–D line lies to the left of the Y-axis. It will be shown later that the length of I indicates the amount of the postulated inhibitor.

Fig. 3 shows that the prolongation of t_{min} must be regarded as a result of vitamin K deficiency rather than as an effect of the presence of circulating Dicumarol; essentially the same results are obtained under anticoagulant treatment and in absolute vitamin K deficiency due to proven resorption defects. Figs. 4 and 5 show that addition of cirrhosis plasma to Dicumarol plasma or vitamin K deficient plasma tends to lower the thrombotest times and therefore the t_{min} of the latter two plasmas. This is the case even though the cirrhosis plasma shows slightly *lower* levels of factors II, VII, IX, and X in the specific test (Table 1). Fig. 6 shows that Dicumarol and vitamin K deficient plasmas do not correct each other's prolongation of t_{min} because t_{min} of the mixture is not lower than the mean of the t_{min}'s of the individual plasmas.

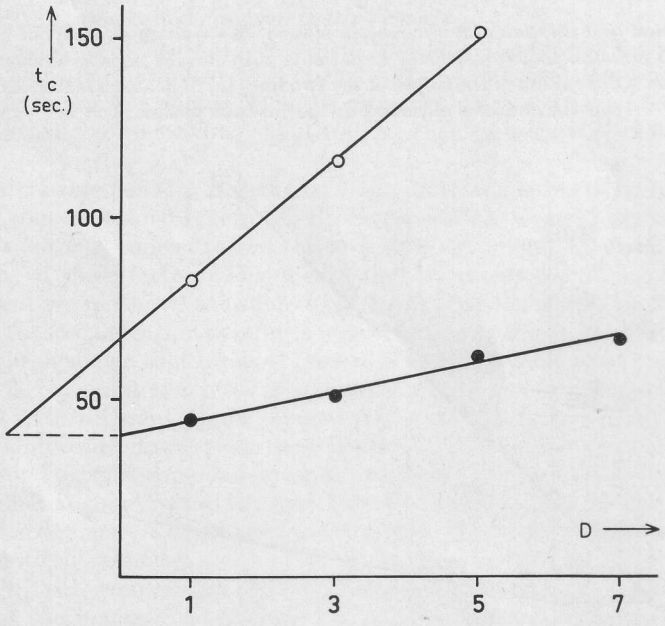

Fig. 3. *Thrombotest-dilution plot of plasma of vitamin K-deficient patients.* ●——● t–D plot of normal plasma; ○——○ t–D plot of pooled plasmas of 7 vitamin K-deficient patients. t_{min} of normal plasma is 39 sec; t_{min} of vitamin K-deficient plasmas of 67 sec. The relation of the slopes indicates a factor X level of 24% in the vitamin K-deficient plasma. I = 1.6.

A frequency distribution curve of the value of I in hepatic disorders, Dicumarol treatment, and vitamin K deficiency is shown in Figs. 7, 8, and 9. These figures clearly show that prolongation of t_{min} and therefore a positive value of I is a feature of absolute as well as drug-induced (relative) vitamin K deficiency.

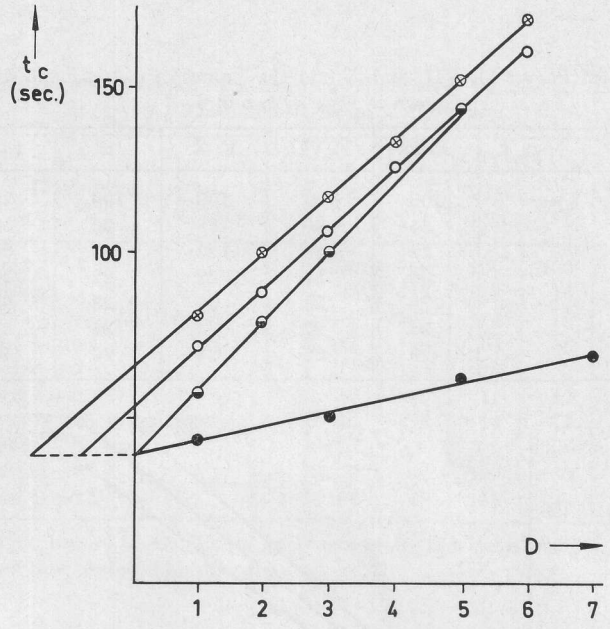

Fig. 4. *Thrombotest-dilution plot of a mixture of cirrhosis plasma and Dicumarol plasma.* ●———● t–D plot of normal plasma; ◒———◒ t–D plot of cirrhosis plasma; ⊗———⊗ t–D plot of Dicumarol plasma. ○———○ t–D plot of a mixture of equal parts of cirrhosis plasma and Dicumarol plasma. For the values of t_{min}, α, and I, see Table 1.

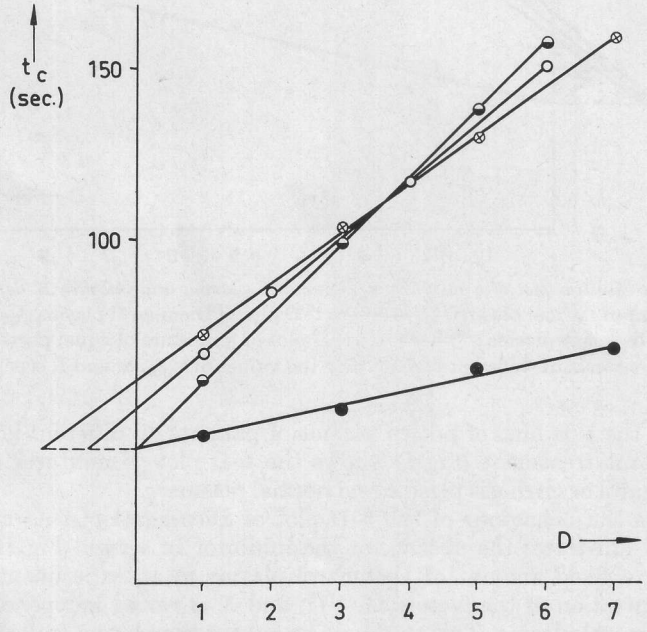

Fig. 5. *Thrombotest-dilution plot of a mixture of cirrhosis plasma and vitamin-K deficient plasma.* ●———● t–D plot of normal plasma; ◒———◒ t–D plot of cirrhosis plasma; ⊗———⊗ t–D plot of vitamin K-deficient plasma; ○———○ t–D plot of a mixture of equal parts of cirrhosis plasma and vitamin K-deficient plasma. For the values of t_{min}, α, and I, see Table 1.

Table 1. The Level of Factor II, VII, and X and the Values of α, t_{min}, and I of the Plasmas Shown in Figs. 4, 5, and 6.

Plasma	F. II	F. VII	F. X	α	t_{min}	I
Normal	100	100	100	100	39	0
Dicumarol	34	30	28	30	59	1.4
Vitamin K-def.	22	23	23	26	66	1.6
Cirrhosis	30	27	22	22	39	0
Dic./vit. K-def.	28	23	27	28	62	1.5
Dic./cirrh.	34	27	24	26	51	0.7
Vit. K-def./cirrh.	29	25	24	25	54	0.8

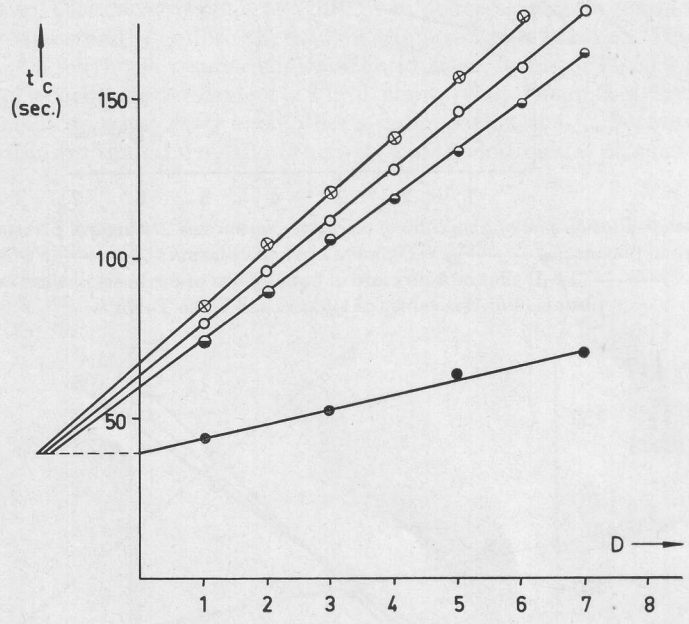

Fig. 6. *Thrombotest-dilution plot of a mixture of Dicumarol plasma and vitamin K-deficient plasma.* ●——● t–D plot of normal plasma; ⊗——⊗ t–D plot of Dicumarol plasma; ◒——◒ t–D plot of vitamin K-deficient plasma; ○——○ t–D plot of a mixture of equal parts of Dicumarol plasma and vitamin K-deficient plasma. For the values of t_{min}, α, and I, see Table 1.

Fig. 10 shows the t–D lines of pooled plasma of patients on different levels of long-term anticoagulant treatment. Fig. 11 shows the t–D plot of mixtures of $Al(OH)_3$-adsorbed Dicumarol or cirrhosis plasma and normal plasma.

Table 2 shows the behaviour of the t–D plot of Dicumarol plasma after various treatments, and illustrates the absence of the inhibitor in serum. Fig. 12 shows the effect of adding a fixed amount of Dicumarol plasma in an experimental situation when the concentration of the factors II, VII, and X is varied independently of the amount of Dicumarol plasma. This was done by taking the plasma (called plasma A), of an anticoagulated patient (factor II: 10%; factor VII: 9%; factor X: 10%; factor IX: 11%; I: 2.0) and treating a portion of it with $Al(OH)_3$, after which all factors were absent, as well as the inhibitor, as can be judged from a similar experi-

Table 2. The Influence of Various Treatments of Dicumarol Plasma on the Parameters of its Thrombotest-Dilution Curve.

No.	Description	Clotting factors (%)					t–D plot	
		II	V	VII + X	VII	X	α	I
A	Normal plasma	100	100	100	100	100	100	0
B	Dicumarol plasma	15	115	18	16	18	17	1.5
C	B after adsorption onto $Al(OH)_3$	0	100	0	0	0	—	—
D	Equal parts of A and C	45	100	54	52	50	50	0
E	Equal parts of B and C	7	100	8	9	9	9	0.8
F	B after heating 4 h at 45° C	10	10	12	12	14	13	0.1
G	B after dialysis 24 h at 4° C against Michaelis buffer	15	72	14	15	15	16	1.1
H	Dicumarol serum	2	2	120	150	90	—	—
I	Equal parts of A and H	60	48	115	120	98	93	0.2

Adsorption with $BaSO_4$ or $Ca_3(PO_4)_2$ gave essentially the same results as adsorption with $Al(OH)_3$ provided that oxalated plasma was used.

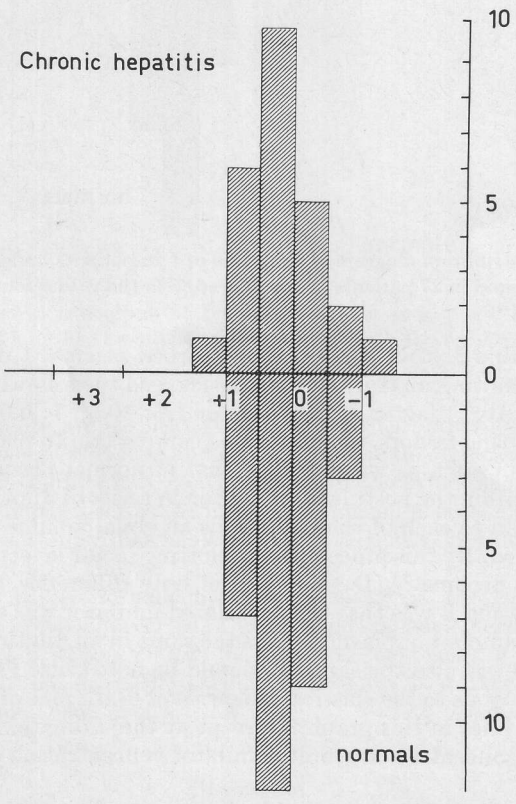

Fig. 7. Frequency distribution diagram of the value of I in patients with chronic hepatitis as compared to normals. The values found in 25 patients are compared with the values found in 31 normals.

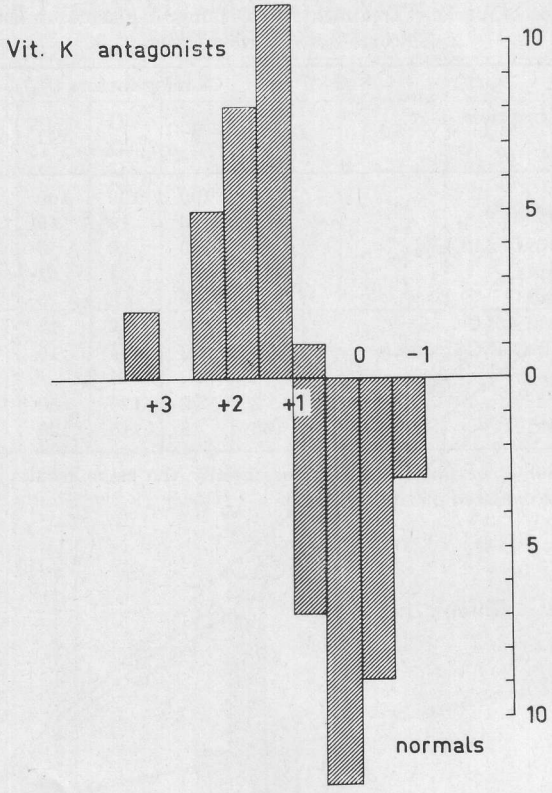

Fig. 8. Frequency distribution diagram of the value of I in patients under 'Dicumarol' treatment. The values found in 27 patients are compared with the values found in 31 normals.

ment shown in Table 2. To 9 parts of the adsorbed plasma, 1 part of normal plasma was added. The resulting mixture (plasma B) showed the following features: factor II: 10%; factor VII: 10%; factor X: 9%; factor IX: 10%; I: 0.0. With respect to the content of the clotting factors, plasma B was comparable to plasma A; but the value 0.0 for I indicates that t_{min} was equal to that of normal plasma and thus that this plasma did not contain the postulated inhibitor. A series of dilutions of normal plasma was then made, and to each of these dilutions an equal volume of either plasma A or plasma B was added. By this procedure the clotting factor level of a dilution of normal plasma (say D%) became $^1/_2(D + 10)\%$. The only difference between the addition of plasma A or plasma B was that the postulated inhibitor was added with A, but not with B. Since the amount of plasma A was the same in all dilutions made, the amount of inhibitor added was also the same. It should be noted that Fig. 10 is *not* a t–D plot but is closely analogous to the classical Lineweaver-Burk plot of enzyme kinetics. The fact that the two lines in this graph intercept at the ordinate, indicates here that at infinite substrate concentration a finite inhibitor concentration does not influence the reaction rate.

Fig. 13 shows the t–D curve produced when a non-competitive inhibitor is present in the plasma, in this case fibrinogen breakdown products. Fig. 14 shows a t–D curve of plasma from a patient with cirrhosis of the liver who was on anticoagulant therapy.

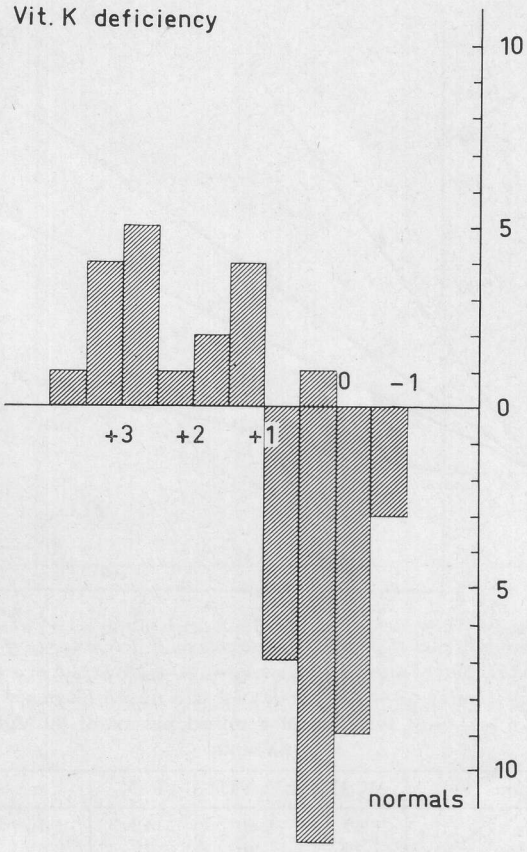

Fig. 9. Frequency distribution diagram of the value of I in patients with proven vitamin K-deficiency. The values found in 18 patients are compared with the values found in 31 normals.

In Fig. 15 experiments are displayed in which the thrombotest-time and the factor X concentration were estimated and compared. The thrombotest-time was plotted against the inverse of the factor X concentration of the same plasma. Two groups of plasmas were tested, firstly a series of mixtures of normal plasma and $BaSO_4$ adsorbed plasma (lower lines), secondly plasmas of different patients on different levels of anticoagulant treatment (upper lines).

It is seen that at the same concentration of factor X the thrombotest-time in Dicumarol plasma is always higher than in (diluted) normal plasma. At infinite factor X concentration (i. e. at the Y-axis) the difference between normal and Dicumarol plasma is no longer found, again indicating the presence of a fixed amount of a competitive inhibitor in the Dicumarol plasma.

Discussion

The curves shown in Figs. 1, 2, 3, 7, 8, and 9 indicate that the two parameters of the t–D line (i. e. slope and t_{min}) vary in vitamin K deficiency but that only one para-

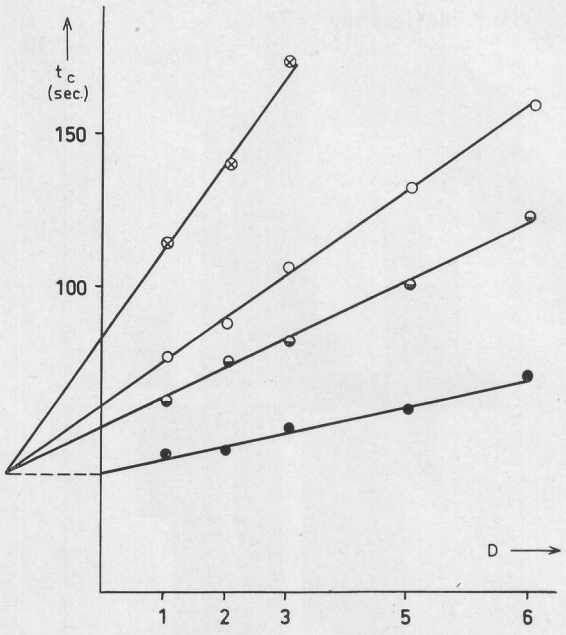

Fig. 10. *Thrombotest-dilution plot of plasmas of patients on different levels of long-term anticoagulant treatment.* ●——● t–D plot of normal plasma; ◒——◒ t–D plot of a mixed plasma of 15 low-level anti-coagulated patients; ○——○ t–D plot of a mixed plasma of 15 medium-level anti-coagulated patients. ⊗——⊗ t–D plot of a mixed plasma of 30 high-level anti-coagulated patients.

Level of anticoagulation	F. II	F. VIII	F. X	α	t_{min}	I
Low	47	50	42	46	50	1,6
Medium	32	30	27	31	62	1,6
High	12	15	13	16	83	1,6

meter (i. e. slope) varies in chronic liver disease. A marked elevation of t_{min} is seen only in absolute or drug induced vitamin K deficiency.

It appears to make no difference whether the vitamin K deficiency is induced by drugs or caused by defective absorption (Fig. 6). The changes seem to be quantitatively related to the amount of vitamin K-deficient plasma present (Figs. 4 and 5).

In accordance with the conclusion drawn in our theoretical considerations above, the change in t_{min} must be due to the presence or absence of a modifier. This modifier cannot be one of the known clotting factors, since t_{min} is a clotting time at infinite factor X concentration and factors II and VII have been shown not to be rate-limiting in this situation (9), factor IX does not influence the thrombotest time, and no other known clotting factors are affected by vitamin K deficiency. This leaves us with four possibilities:

a) an abnormal procoagulant is present in chronic liver disease but not in vitamin K deficiency;

b) a normal inhibitor is absent in chronic liver disease but present in vitamin K deficiency; (N. B. This could *not* be Antithrombin 3 because this inhibitor is present in excess in the Thrombotest reagent.)

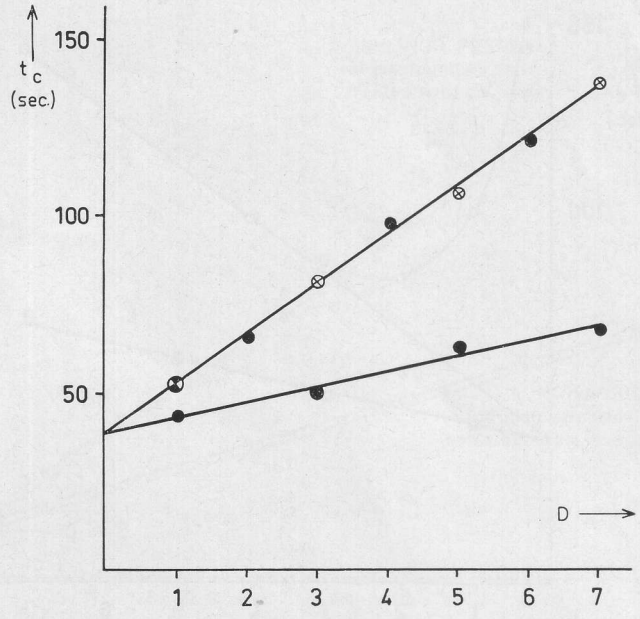

Fig. 11. *Thrombotest-time dilution plot of a mixture of normal and Al(OH)$_3$ adsorbed Dicumarol plasma or cirrhosis plasma.* ●———● t–D plot of a normal plasma; ●———● (upper curve) t–D plot of a mixture of Al(OH)$_3$-adsorbed cirrhosis plasma (7 parts) and normal plasma (3 parts). ⊗———⊗ t–D plot of a mixture of 3 parts normal plasma and 7 parts Al(HO)$_3$-adsorbed Dicumarol plasma.

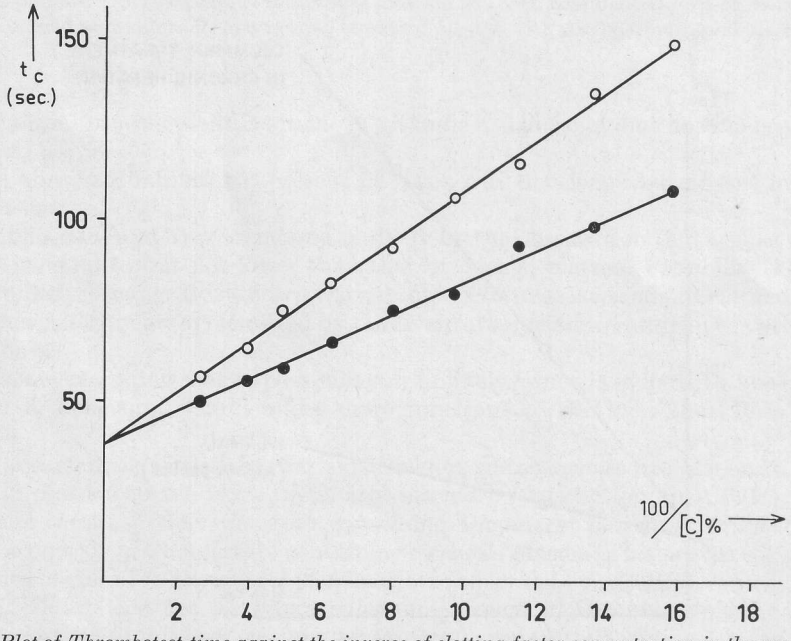

Fig. 12. *Plot of Thrombotest-time against the inverse of clotting-factor concentration in the presence and absence of a fixed amount of Dicumarol plasma.* Explanation in text.

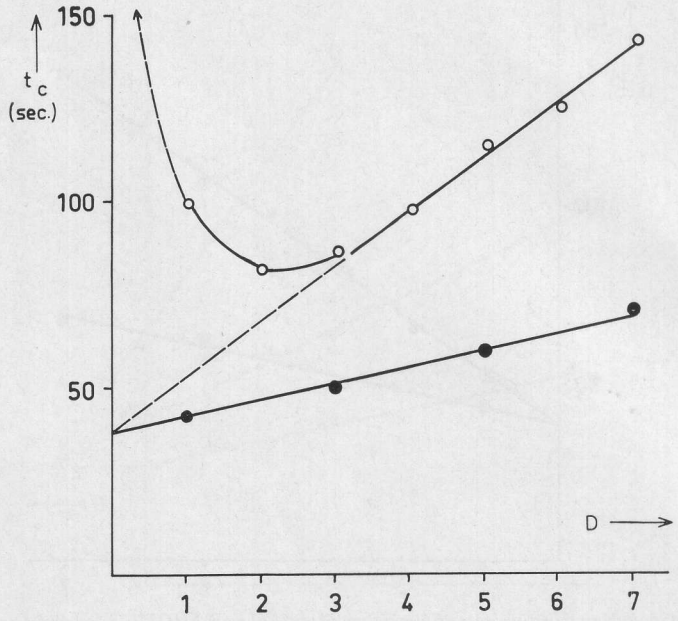

Fig. 13. *Thrombotest-time dilution plot in the presence of a non-competitive inhibitor.* ●——● normal plasma; ○——○ pathological plasma.

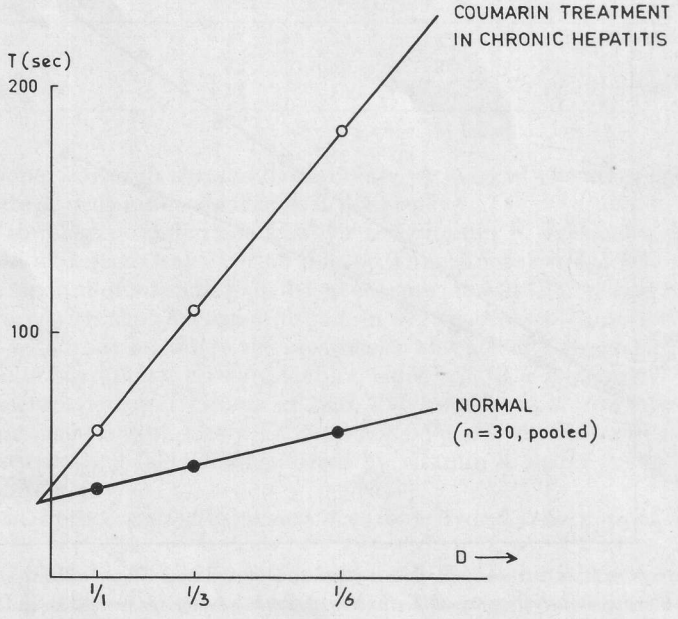

Fig. 14. *Thrombotest-time dilution in plot after 'Dicumarol' administration in a case of liver cirrhosis.*

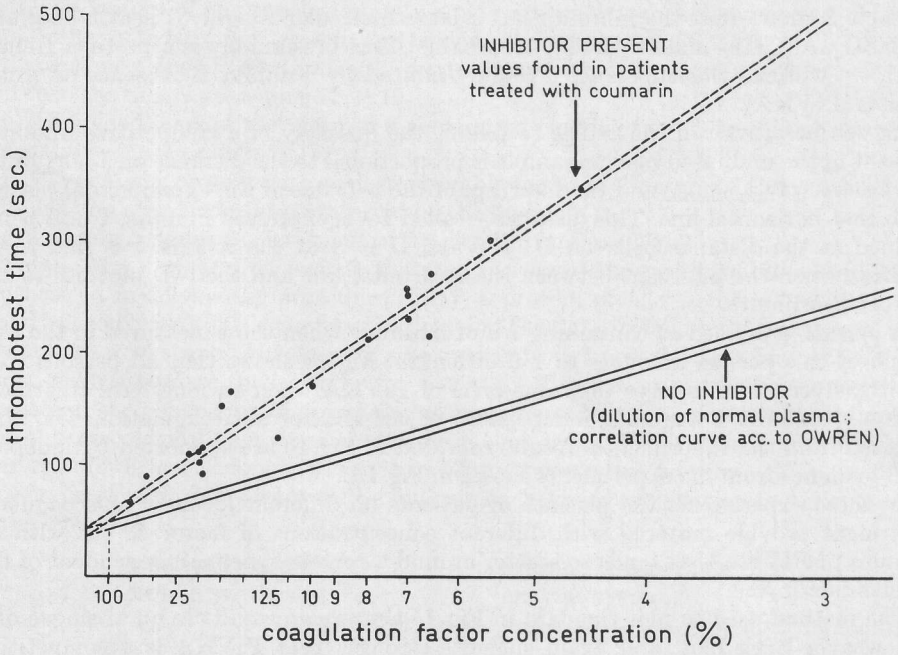

Fig. 15. *Relation between factor X concentration and thrombotest-time in Dicumarol plasmas from different patients.* The reciprocal of factor X concentration (X axis) is plotted against thrombotest-time (Y axis) obtained with the same plasma. Each dot represents values obtaned with plasma of a different patient. The two parallel lower lines indicate the 85% confidence limits of the same data obtained with dilutions of a normal standard plasma with $Al(OH)_3$-adsorbed plasma.

c) a normal procoagulant is absent in vitamin K deficiency but not in chronic liver disease;

d) an abnormal inhibitor is present in vitamin K deficiency but absent in chronic liver disease.

Possibilities a) and b) are rendered unlikely by the observation that t_{min} of cirrhosis plasma is equal to but not lower than that of normal plasma. Possibility a) is even more unlikely because it is known that chronic liver disease tends to cause depletion of plasma constituents synthesized by the liver rather than to result in the production of new ones.

The same reasoning makes possibility c) unlikely, since it is hard to imagine that vitamin K deficiency would affect more functions of the liver than does chronic cirrhosis.

The argument is settled by the experiments whose results are shown in Fig. 11. Removal of a substance from Dicumarol plasma by adsorption on $Al(OH)_3$ returns to normal the t_{min} of the mixture containing Dicumarol plasma whereas it has no influence on t_{min} of the mixture containing cirrhosis plasma. This proves the presence of an anticoagulant in Dicumarol plasma that is adsorbed by $Al(OH)_3$.

Fig. 12 shows that the inhibitory substance present in Dicumarol plasma behaves as a competitive inhibitor: at infinite substrate concentration (i. e. at the Y-axis) the presence of a *fixed amount* of inhibitor has no influence on the clotting time.

Table 2 shows that the inhibitor has a large molecular weight, is heat labile; and is $BaSO_4$, $Al(OH)_3$- and $Ca_3(PO_4)_2$-adsorbable; thus presumably is a protein. Henceforth we will call this substance *P*rotein *I*nduced by *V*itamin *K* *A*bsence or *A*ntagonists (PIVKA).

As will be proven in the article to follow, the quantity of a competitive inhibitor present in the undiluted plasma sample is proportional to the distance on a horizontal line between $t_{min.\,uninh.}$ and the intercept of the t–D line of the experimental plasma with that horizontal line. This distance – called I – is expressed in units, 1 unit being defined as the distance between D = 0 and D = 1 at the X-axis. I is said to be positive when the intercept between the horizontal line and the t–D plot lies to the left of the ordinate.

A plasma is defined as containing 1 u of inhibitor when the I measured in the t–D graph of this plasma amounts to 1 u of length. Fig. 10 shows that all persons with healthy livers tend to have the same level of PIVKA when on long-term treatment with a vitamin K antagonist, independent of the level of anticoagulation. Our conclusions from the experimental results reproted in Fig. 10 are supported by independent evidence from the experiments shown in Fig. 15.

In these experiments the plasmas of patients on different levels of anticoagulant treatment provide material with different concentrations of factor X but with an amount of PIVKA that tends to scatter around a constant mean, independent of the level of factor X.

The method used to plot the data in Fig. 15 thus again results in an analogue of a Lineweaver-Burk plot, and again suggests strongly that PIVKA is a competitive inhibitor of a reaction involving factor X.

Fig. 14 shows an observation concerning accidental Dicumarol administration in a case of liver cirrhosis. This result suggests that in this case there was hardly any synthesis of the inhibitor, which might be a first indication that PIVKA is synthesized in the liver. The adsorption characteristics indicate a protein that has much in common with the clotting factors of the so-called "prothrombin complex" i. e. the clotting factors II, VII, IX, and X (Table 2). The fact that we deal with a competitive inhibitor of thrombin formation suggests that the substance may be a substrate analogue of one or more of the clotting factors II, VII, IX, or X. This again suggests a protein closely resembling one or more of these factors. In view of the known difficulties of separating the prothrombin complex into its constituent factors (2, 30), it is not surprising to see that it is possible to achieve a considerable purification of the inhibitor relative to plasma proteins but that it is difficult to separate it from the factors of the prothrombin complex. Extensive column chromatographic experiments are being carried out in our laboratory at present, (J. M. C. Wimmers and A. C. W. Swart) they occasionally show fractions with an inhibitory action only. The identity of this protein and the inhibitor postulated here has not yet been confirmed, however. Nevertheless, we think that the kinetical approach described here yields enough evidence to recognize PIVKA as a distinct entity. The question of why vitamin K deficiency would induce the synthesis of an inhibitor cannot be solved at the moment, but the known facts provide a basis for the construction of a hypothesis which can serve as a rewarding tool for the design of further experiments.

The following facts are to be recognized:

a) There is no known influence of vitamin K upon protein synthesis as such (25);

b) A specific influence of vitamin K on the protein synthesis of some clotting factors has been suggested (20). However, a close examination of these experiments shows that they do not present any discrepancies with the hypothesis put forward here (21);

c) The factors of the prothrombin complex are known to be conjugated proteins containing up to 50% non-protein material, mostly sugars (16, 26, 31);

d) Glycoproteins are synthesized in two steps; the protein moiety is produced first and then the sugars are attached (4, 19);

e) The blood level of the vitamin K dependent clotting factors is kept constant by the activity of liver cells;

f) PIVKA is a protein with a close resemblance to the factors of the prothrombin complex and appears to be synthesized by the liver;

g) Vitamin K is not a constituent of the clotting factors (27).

Points a), b), c), and d) suggest that the factors of the prothrombin complex are synthesized in a two-step procedure. First, a protein moiety is made and then this moiety is converted into a conjugated protein. The data in the literature suggest that vitamin K works upon the second step rather than upon the first one. We do not know what comprises the second step, but as an illustration we shall assume that it consists of the conversion of a protein into a glycoprotein. The structural similarities and pharmacological behaviour of vitamin K and drugs of the cumarol group suggest that vitamin K is a coenzyme in a system that catalyses this protein ------→ glycoprotein conversion. This leads to the following scheme of coagulation factor synthesis in the liver cells:

	genetically-controlled protein synthesis		vitamin K-sensitive enzyme system	
amino acids	----------→	protein	----------→	clotting factor

The fact that the clotting factor concentration in the plasma is maintained at a constant level suggests a feedback system in which the level of a clotting factor controls its synthesis rate. The controlled rate could be the rate of either the first or the second step. If the rate of the first step were under the control of this feedback mechanism inhibition in the second step would cause maximal synthesis in the first step. The product of this first step, would only be converted into the clotting factor slowly because of the inhibition of the second step. This would lead to piling up of the intermediate product. If this product was able to reach the circulation, its presence there would explain the observed facts, since the intermediate product is a protein much like the final clotting factor synthesized by the liver. It therefore could very well be a substrate analogue of one of the clotting factors and thereby an inhibitor.

The constant level found for steady-state anticoagulation in patients with a healthy liver represents a maximal synthesis rate in the first step combined with a steady low rate of conversion in the second step. Isolation of the inhibitor would offer a unique opportunity for the study of the vitamin K-dependent enzyme system in liver cell fractions.

Summary

Application of enzyme kinetics to the results of thrombotest estimations in correlation with specific clotting factor estimations has led to the recognition of a protein moiety that occurs in plasma in vitamin K deficiency and acts as a competitive inhibitor of thrombin formation. A hypothesis is given by which the occurrence of this inhibitor is explained in terms of a biphasic synthesis of the vitamin K-dependent clotting factors.

Résumé

La théorie de la cinétique enzymatique appliquée aux résultats de l'estimation du Thrombotest en corrélation avec l'estimation spécifique des facteurs de la coagulation conduit à la reconnaissance d'une protéine plasmatique dans les cas de déficiences en vitamine K, qui agit comme un inhibiteur compétitif de la formation de la thrombine. Une hypothèse est émise suivant laquelle l'apparition de cet inhibiteur serait expliquée par la synthèse en 2 temps des facteurs de la coagulation dépendant de la vitamine K.

Zusammenfassung

Die Anwendung von Enzymkinetik auf die Ergebnisse der Thrombotestbestimmungen in Beziehung mit spezifischen Gerinnungsfaktorbestimmungen hat zu der Erkennung eines Eiweißstoffes geführt, der im Plasma bei Vitamin-K-Mangel vorkommt und als ein kompetitiver Hemmstoff der Thrombinbildung wirkt. Es wird eine Hypothese aufgestellt, welche das Auftreten dieses Hemmstoffes in Form einer 2-phasischen Synthese der Vitamin-K-abhängigen Gerinnungsfaktoren erklärt.

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